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Angiopep-2-Mediated Delivery of Human Manganese Superoxide Dismutase in Brain Endothelial Cells and its Protective Effect Against Oxidative Stress

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Abstract Oxidative stress has been considered as the leading cause of blood–brain barrier disruption which implicates many neurological disorders. Manganese superoxide dismutase (MnSOD or SOD2) is one of the crucial antioxidant enzymes that can provide substantial protection against oxidative damage. However, the therapeutic effect of the enzyme in such neurological diseases is limited due to poor transduction into brain microvascular endothelial cells. In the present study, a fusion protein of human SOD2 and a brain targeting peptide, Angiopep-2 (AP-2), was generated by genetic engineering. The SOD2-AP-2 and control SOD2 were successfully expressed in *Escherichia coli* and purified using immobilized metal affinity chromatography. Purified SOD2-AP-2 exhibited 1,090 μ/mg of specific SOD activity, which retained a significant activity in the same order of magnitude as that of native SOD2. The in vitro transduction demonstrated that 1 μM of SOD2-AP-2 delivered efficiently to immortalized mouse brain endothelial cell line within 30 min whereas, control SOD2 did not. Moreover, pretreatment with 50 units of SOD2-AP-2 for 1 h could significantly protect cells against paraquat up to 2 mM but control SOD2 pretreatment did not show a protective effect. Taken together, our findings pave the way for SOD2-AP-2 to be a potential therapeutic candidate for neurological diseases.

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Abbreviations

AP-2	Angiopep-2
TAT	HIV-1 TAT protein transduction domain

Introduction

The blood–brain barrier (BBB) is a physical and metabolic barrier formed by brain microvascular endothelial cells (BMVECs) that maintains brain homeostasis and restricts the passage of substances from the blood circulation to the brain (Hawkins and Davis 2005). Results from clinical and animal model studies have revealed that BMVECs death leading to loss of BBB integrity is critical for the development and progression of neurological disorders (Chrissobolis et al. 2011; Kaur et al. 2011; Kortekaas et al. 2005; Minagar and Alexander 2003; Pan et al. 2007; Petito and Cash 1992; Zipser et al. 2007). Free radicals have been suggested to involve substantially in BBB breakdown (Abbott et al. 1992). Although BMVECs usually possess high levels of intracellular antioxidant defense systems, they can be altered leading to progressive oxidative damage during various disease states and aging (Betz 1993; Tayarani et al. 1989). Such damage increases vascular endothelial permeability and promotes leukocyte activation resulting in BBB disruption (Lum and Roebuck 2001). To alleviate this deleterious effect, the effective therapeutic strategies should be targeted toward the BMVECs.

Several studies have focused on the utilization of various antioxidant enzymes as protective agents (Fennell et al. 2002;

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He et al. 2006; Hood et al. 2014; Uyama et al. 1992). Superoxide dismutase (SOD) is an endogenous enzyme that is a part of the first line of defense through elimination of O_2^- by converting it into H_2O_2 and O_2. The enzyme has been considerable interested because of its role in protection against human diseases (Faraci and Didion 2004; Fukai and Ushio-Fukai 2011). Manganese superoxide dismutase (MnSOD or SOD2) is one of the most crucial antioxidants in the central nervous system (CNS) and is thought to be one of the major mechanisms to counteract injuries of reactive oxygen species (ROS) after cerebral ischemia (Huang et al. 2012; Jung et al. 2009; Kim et al. 2002; Lebovitz et al. 1996). The comparative pharmacokinetic studies in rats demonstrated that MnSOD had longer serum half-life than Cu/ZnSOD indicating the superior advantage of MnSOD for the treatment of chronic diseases (Baret et al. 1984; Gorecki et al. 1991). Altogether, MnSOD is promising to be a potential therapeutic enzyme for neurological disorders. However, lack of transduction ability of the enzyme into target cells, particularly BMVECs that possess few fenestrae and few endocytic vesicles compared with the endothelial cells of other organs, limits its effectiveness for therapeutic application (Pardridge 1999).

Currently, the protein transduction technology is widely used to deliver a range of proteins into mammalian cells. To deliver an exogenous protein into cells, small regions of protein, called protein transduction domains (PTDs) or cell penetrating peptides (CPPs), have been developed (Wadia and Dowdy 2002). One of the most well known PTDs is TAT peptide which is derived from HIV-1 trans-activator of transcription protein (Watson and Edward 1999). It is composed of 11 amino acids (YGRKKRRQRRR). When native proteins were fused with TAT by genetic engineering, the fusion proteins could be transduced across the cell membrane while maintaining their activities (Eum et al. 2004; Jin et al. 2001). The TAT-fusion proteins were shown to transduce into all mammalian cells and tissues, including those present across the BBB (Schwarze et al. 1999; Schwarze and Dowdy 2000). Since TAT peptide lacks of cell selectivity and can enter to any cell types, the alternative CPPs with high specificity have been used for delivery. Recently, a novel brain targeting peptide namely Angiopep-2 (AP-2) has been designed and applied for brain targeting delivery. This synthetic peptide consists of 19 amino acids (TFFYGGSRGKRNNFKTEEY) which is derived from the Kunitz protease inhibitor (KPI) domain. This peptide binds to a specific receptor, low-density lipoprotein receptor-related protein-1 (LRP1), which is highly expressed on BMVECs of the BBB (Demeule et al. 2008a, 2008b). Because of its higher transcytosis capacity compared with other brain targeting proteins, such as transferrin and aprotinin, AP-2 has been used as a peptide-based delivery system that provides a non-invasive and flexible platform for transporting drugs or biological active molecules across the BBB into the CNS (Demeule et al. 2008a; Bertrand et al. 2010).

In this study, the human SOD2 and AP-2 (SOD2-AP-2) fusion protein was engineered and purified, then its SOD activity was determined. The transduction ability and protective effect against paraquat-induced oxidative stress were investigated. Moreover, the purified control SOD2 protein and SOD2-TAT fusion protein were also produced and included in all experiments for comparison. Herein, the immortalized mouse brain endothelial cell line (bEnd.3) was utilized since it has been established as an in vitro BBB model that preserves features of the in vivo brain endothelium (Li et al. 2010; Tyagi et al. 2009). Our findings indicate that SOD2-AP-2 efficiently transduced into bEnd.3 cells and provided a protective effect against oxidative stress in vitro leading us to suggest that it may be a potential therapeutic agent for neurological disorders associated with BMVECs damage and BBB dysfunction.

Materials and Methods

Construction of SOD2-AP-2 and SOD2-TAT

Cloning of a gene encoding human SOD2 (hMnSOD; NCBI accession number Y00472) into pET46 was performed as previously described (Yainoy et al. 2007). To construct a SOD2-AP-2 fusion gene, the stop codon of SOD2 gene was changed to *Hind*III restriction site by Quikchange site-directed mutagenesis kit (Agilent Technologies, USA). The AP-2 oligonucleotide was synthesized to contain *Hind*III and *Xho*I at 5′ and 3′ ends, respectively. To generate pET46-SOD2-AP-2, an open reading frame encoding the AP-2 peptide was cloned into the *Hind*III and *Xho*I sites of the mutagenized pET46-SOD2. The HIV-1 TAT transduction domain was also cloned into the pET46-SOD2 using the same strategy to generate the pET46-SOD2-TAT. The constructs were verified by DNA sequence analysis.

Expression and Purification of SOD2-AP-2 and SOD2-TAT

The recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) (Novagen). The transformants were cultured in terrific broth medium with 100 μg/ml ampicillin at 37 °C, 150 rpm until OD600 nm = 0.5. Then, isopropyl-β-D-thiogalactoside (IPTG) (Bio Basic Canada Inc., Canada) and $MnCl_2$ (Bio Basic Canada Inc., Canada) were added simultaneously at a final concentration of 1 mM and 200 ppm, respectively to induce protein expression and increase solubility of recombinant protein (Yainoy et al. 2007). The cells were cultured further at 30 °C, 120 rpm for overnight. Cells were collected by centrifugation and suspended in lysis buffer (50 mM phosphate buffer, pH 7.4) followed by sonication. After

centrifugation, the supernatant was filtered through a 0.45 μm filter and purified by immobilized metal affinity chromatography using a Ni–NTA Sepharose column (pre-equilibrated withlysis buffer) with ÄKTA prime protein purification system (GE healthcare life sciences, UK). After elution with gradient imidazole in the same buffer, the target protein containing fractions were combined. The imidazole was removed and the purified proteins were concentrated by spinning the combined fractions through an Amicon Ultra 10,000 MWCO filter (Millipore Corp., USA). Protein molecular weight and purity under denaturing condition were determined by SDS-PAGE analysis. In addition, the purified proteins were also resolved their native molecular weight by 16/60 Sephacryl S-300 HR gel filtration chromatography (GE healthcare life science, UK). Glycerol was added to all purified proteins at a final concentration of 10 % and protein concentrations were measured by the Bradford method (Bradford 1976) before storage at −80 °C. The native SOD2 was expressed and purified using the same system to employ it as a control SOD2.

Measurement of Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was measured according to the inhibition of nitroblue tetrazolium (NBT) reduction by superoxide radicals generating from NADH/phenazine methosulfate (PMS) reaction at aerobic and non-acidic pH conditions as described previously (Grey et al. 2009). The absorbance at 560 nm was monitored during 5 min as an index of NBT reduction using a UV–Vis spectrophotometer, then calculated the enzyme inhibition (%) to define IC_{50} values and specific enzymatic activity.

Cell Culture

The bEnd.3 cell line (ATCC CRL-2299) was cultured in DMEM (Gibco, USA) containing 10 % fetal bovine serum (Gibco, USA) and antibiotics (100 μg/ml streptomycin and 100 μ/ml penicillin). Cells were routinely grown to >80 % confluence in 25 cm^2 flasks at 37 °C in a humidified atmosphere of 5 % CO_2 before passage and seeding for experiments.

Transduction of SOD2-AP-2 and SOD2-TAT

The transduction ability of target proteins was examined by confocal microscopy. Briefly, each purified protein was conjugated with Alexa Fluor 488 dye according to manufacturer's instructions (Invitrogen, USA). The bEnd.3 cells were seeded at 100,000 cells per well in a four-well chamber slide (Thermo Fisher Scientific Inc., Canada) and allowed to grow at 37 °C for 48 h. The culture medium was replaced with DMEM containing 2 % FBS, then bEnd.3 cells were treated with 1 μM of a labeled protein at various time periods. After being washed with PBS, the cells were fixed with 4 % ice-cold paraformaldehyde and mounted in 90 % glycerol. Fluorescence images of cells were monitored and taken (×600 magnification) by Olympus confocal laser-scanning system then, analyzed by FV-10 ASW 2.1 software.

Effects of Transduced SOD2-AP-2 and SOD2-TAT on the Viability of bEnd.3 Cells Treated with Paraquat

Cells were seeded in 96-well microplates at 20,000 cells per well for 12 h, discarded the medium and then pre-treated with or without 50 units of purified proteins for 1 h. After being washed with PBS, the cells were treated with paraquat (methyl viologen, Sigma-Aldrich, USA) at various concentrations for 12 h. Cell viability was measured by a colorimetric assay using Cell Titer 96 Aqueous MTS reagent (Promega, USA) at 490 nm and a microplate reader (Tecan Group Ltd., Switzerland). The experiments were performed in triplicate. The cell viability (%) of test was the relative value as compared to the cell viability (%) of control (without protein and paraquat treatment).

Statistical Analysis

Data are presented as mean ± standard deviation (SD). All results were analyzed for normal distribution using Kolmogorov–Smirnov test (P value < 0.05). When no significant difference from normality was identified, comparison of two means was performed using paired t test. A value of $P < 0.05$ with two-tailed t test was considered statistically significant. All statistical calculations were performed using PASW statistic 18 (SPSS Inc., USA).

Results

Construction, Expression and Purification of SOD2-AP-2 and SOD2-TAT

In this study, pET46-SOD2-AP-2 and pET46-SOD2-TAT were successfully constructed (Fig. 1). The AP-2 and TAT sequences were fused in-frame to C-terminus of the SOD2 protein containing an N-terminal polyhistidine purification tag. All proteins were expressed and purified to homogeneity as shown in Fig. 2. Control SOD2, SOD2-AP-2 and SOD2-TAT fusion proteins exhibited correct molecular mass as compared to the theoretical molecular weight of approximately 24, 26.5 and 26 kDa and their yields were 98, 4 and 10 mg/liters of culture, respectively. Under native condition, they showed their molecular weight of approximately 100 kDa as determined by gel filtration

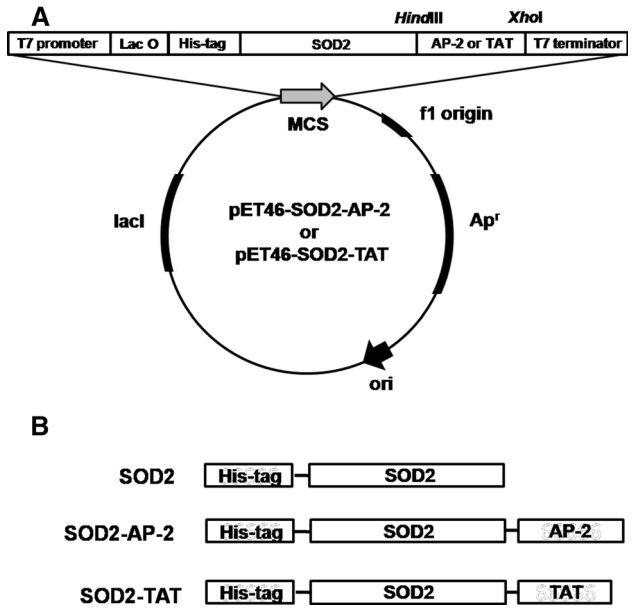


Fig. 1 Construction of SOD2-AP-2 or SOD2-TAT expression vector system based on the vector pET46-SOD2. **a** The coding frame of human SOD2 (599 bp) is located downstream of 6-His tag. The synthetic Angiopep-2 and TAT oligomers were cloned at C-terminus into the *Hind*III and *Xho*I sites of pET46-SOD2 that stop codon of SOD2 was replaced with *Hind*III site by site-directed mutagenesis. The resulting vectors were named pET46-SOD2-AP-2 and pET46-SOD2-TAT. The expression is induced by addition of IPTG. **b** Diagram of expressed control SOD2, SOD2-AP-2 and SOD2-TAT fusion proteins

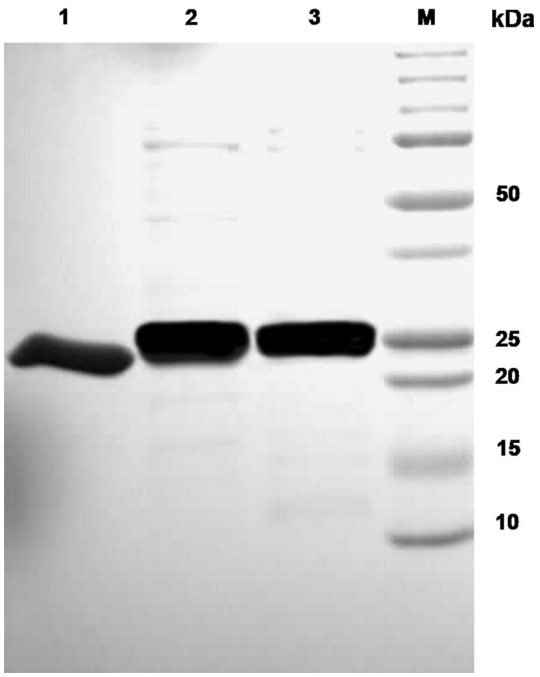


Fig. 2 Expression and purification of control SOD2, SOD2-AP-2 and SOD2-TAT fusion proteins in *Escherichia coli*. Purified proteins were determined by 12 % SDS-PAGE analysis. Lanes are as follows: *lane 1*, purified control SOD2; *lane 2*, purified SOD2-AP-2; *lane 3*, purified SOD2-TAT

chromatography (data not shown). This indicated that each protein forms as tetramers representing the proper molecular conformation.

Measurement of Superoxide Dismutase (SOD) Activity

The specific activity of SOD2-AP-2 and SOD2-TAT was reduced approximately 50 % of control SOD2 (Table 1). Despite the twofold decrease in SOD specific activity, both fusion proteins retained their significant activity in the same order of magnitude as that of control SOD2.

Table 1 Specific and relative SOD activities of control SOD2, SOD2-AP-2 and SOD2-TAT

Protein	Specific SOD activity[a]		Relative SOD activity	
	U/mg	U/μmol	U/mg	U/μmol
Control SOD2	2,270	2.1792×10^5	100	100
SOD2-AP-2	1,090	1.1554×10^5	48.0	53.0
SOD2-TAT	1,015	1.0556×10^5	44.5	48.5

[a] One unit of SOD activity was defined as the amount of enzyme that caused 50 % decrease in NBT reduction

Transduction of SOD2-AP-2 and SOD2-TAT

The transduction ability of fusion proteins into bEnd.3 cells was tested. As shown in Fig. 3, SOD2-AP-2 and SOD2-TAT were efficiently transduced into the cells, whereas control SOD2 was not. The negative control cells and control SOD2 pretreated cells showed weak fluorescence signals, this could be due to auto-fluorescence of the cells. In addition, both fusion proteins rapidly transduced into the cells at 30 min of incubation as determined by confocal microscopy (Fig. 3a). Apparently, the intracellular concentrations of transduced SOD2-AP-2 and SOD2-TAT based on fluorescence signals were comparable. Moreover, the time-dependent transduction of both proteins was not observed during treatments (Fig. 3a–c). Notably, SOD2-AP-2 and SOD2-TAT were distributed mainly in cytoplasm of treated cells over the period of observation.

Effects of Transduced SOD2-AP-2 and SOD2-TAT on the Viability of bEnd.3 Cells Treated with Paraquat

We then investigated whether SOD2-AP-2 and SOD2-TAT protect bEnd.3 cells from paraquat induced toxicity, as an indication that these transduced proteins functioned properly within the cells. As shown in Fig. 4, when cells were treated with various concentrations of paraquat for 12 h, a dose-

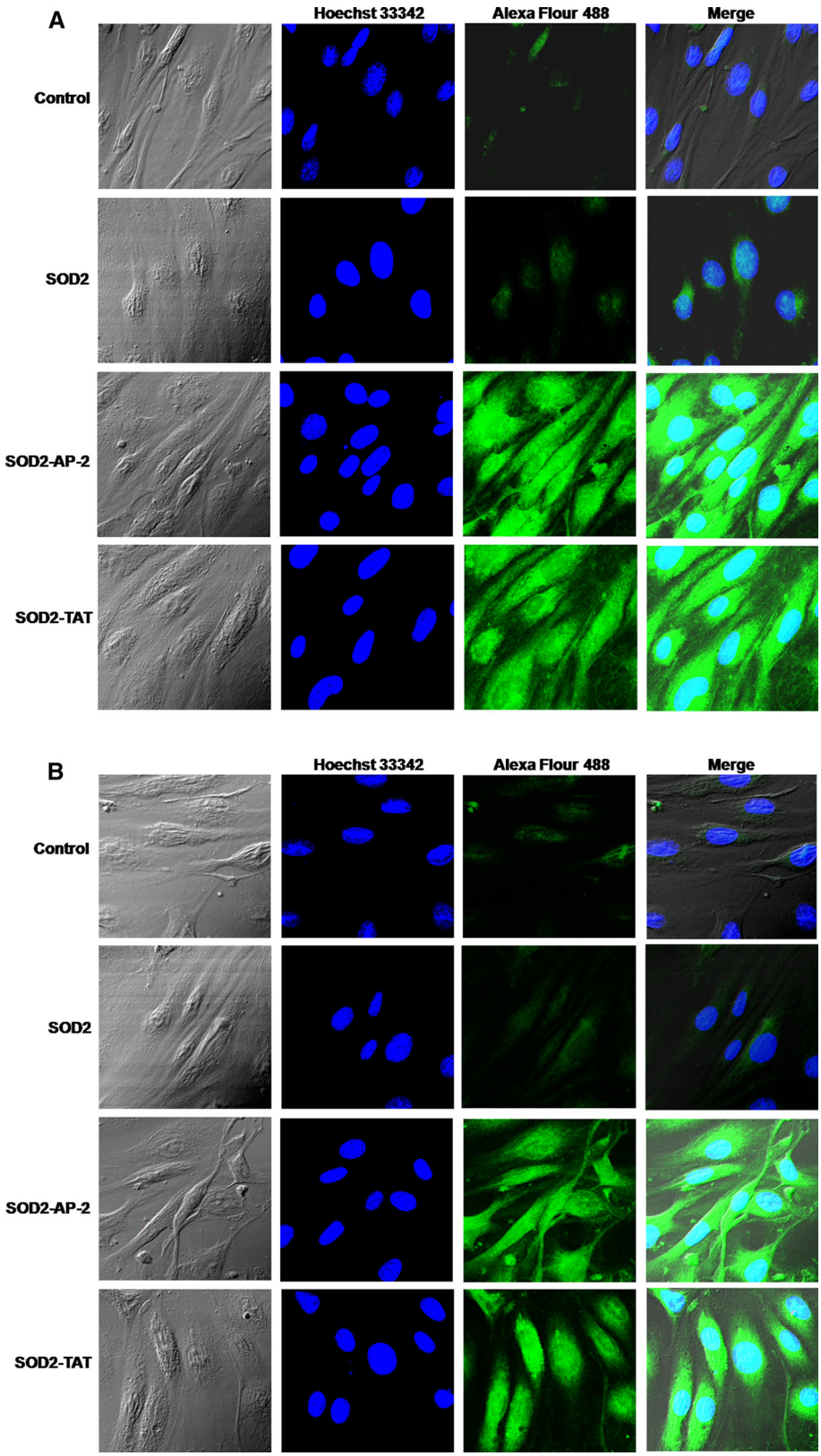
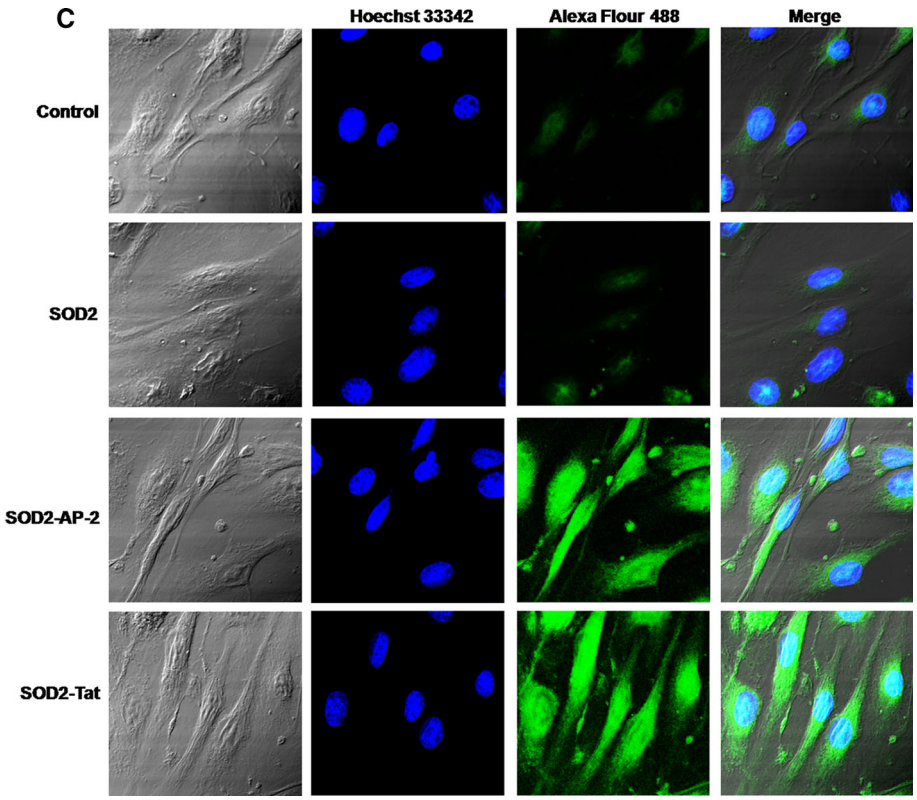


Fig. 3 Transduction of control SOD2, SOD2-AP-2 and SOD2-TAT fusion proteins into bEnd.3 cells. Purified proteins were conjugated with Alexa fluor 488 fluorescent dye and 1 μM of each fluorescent-conjugated protein was added to the culture medium, then cells were incubated at different time points. Cells were washed and fixed with PBS and 4 % paraformaldehyde, respectively. Confocal microscopic images of cells are shown (original magnification, 600×). **a** 30 min, **b** 60 min and **c** 120 min. Control was the cells without protein treatment. *Green fluorescent* signals represent Alexa fluor 488-labeled fusion proteins and *blue fluorescent* signals represent Hoechst 33342-labeled nuclei. Results are representative of two separate experiments (Color figure online)

dependent decrease in cell viability was observed. No cytotoxic effects were observed when cells were pretreated only with proteins under defined conditions (Fig. 4). Pretreatment with control SOD2 for 1 h did not confer a significant protection against all paraquat concentrations when compared with only paraquat treated cells (*P*-

Fig. 3 continued



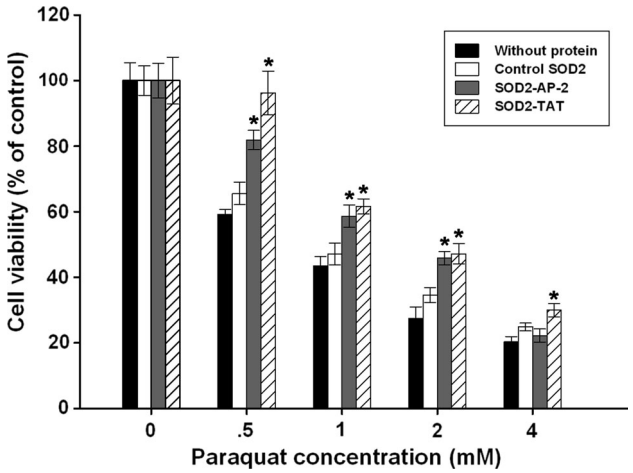


Fig. 4 Effect of transduced fusion proteins on cell viability. The cultured bEnd.3 cells were pretreated with 50 units of control SOD, SOD2-AP-2 and SOD2-TAT fusion proteins for 1 h and then incubated for 12 h in the presence of 0–4 mM paraquat. Cell viability was estimated by a colorimetric assay using MTS. $*P < 0.05$ compared with paraquat treated cells (*without protein*). Each *bar* represents the mean ± SD obtained from three experiments. The statistical analysis was evaluated by paired *t* test.

value < 0.05). However, the cell viability was significantly increased when pretreated with 50 units of SOD2-AP-2 in the presence of paraquat up to 2 mM. Interestingly, pretreatment with 50 units of SOD2-TAT showed a significant increase in cell viability at all concentrations of paraquat (Fig. 4). This indicates that transduced SOD2-AP-2 and SOD2-TAT provide the effective protection for bEnd.3 cells against intracellular oxidative stress. However, it should be noted that SOD2-TAT was more protective than SOD2-AP-2 at high dose of paraquat (up to 4 mM).

Discussion

Blood–brain barrier disruption has been considered as a key step of the disease process in many neurological disorders (Sano et al. 2010). One of the major factors of BBB breakdown involves in BMVECSs damage induced by excessive ROS. Administration of exogenous antioxidant enzymes is an alternative therapy for the above mentioned condition. However, the limitation of effective use is an inability of the enzymes to transduce into BMVECs due to their specialized characteristics. Recently, an AP-2 peptide was developed for brain targeting delivery of therapeutic molecules to cross BBB. It specifically binds to the LPR1 on BMVECs and enhances the transduction and transcytosis of cargo molecules, such as placlitaxel loading nanoparticles to the brain resulting in increase of brain accumulation (Xin et al. 2012). In this study, we firstly demonstrate the delivery of recombinant human SOD2 to brainendothelial cells using an AP-2 transduction approach resulting in protection of cells against paraquat. In addition,

SOD2-TAT fusion protein was also genetically engineered for the first time and investigated the BMVECs transduction ability and protective effect against paraquat comparing with SOD-AP-2. It should be noted that SOD2 in our study did not contain a mitochondrial pre-sequence (24 amino acids) at the N-terminus for better enzymatic activity and protein folding as previously described (Yainoy et al. 2007). The result showed that the specific activity of control SOD2 was close to that of the native human MnSOD as reported by others (Yainoy et al. 2007; McCord et al. 1977). However, SOD2-AP-2 and SOD2-TAT showed about twofold decrease in SOD activity. Previously, the crystal structure studies revealed that human MnSOD is a homotetramer whicheach monomer consists of 198 amino acids and is divided into N-terminal helices and a mixed C-terminal α/β domain (Borgstahl et al. 1992; Wagner et al. 1993). Moreover, many site-specific mutagenesis studies indicated that several residues in C-terminal domain associate with the interactions at the active site as well as the interactions at dimer interface that shown to be important for SOD activity (Borgstahl et al. 1992; Sheng et al. 2013; Trinh et al. 2008; Wagner et al. 1993). Therefore, decrease of enzymatic activity in our case could be probably due to the disturbance of the C-terminal fused peptides at active site or dimer interface.

Examination by confocal microscopy revealed that both SOD2-AP-2 and SOD2-TAT successfully transduced into cells with comparable ability and both were found to locate mainly in the cytosol as shown the intense fluorescence signals at 30, 60 and 120 min of incubation period. The localization of fused peptides was consistent with former studies. The TAT fusion proteins have been shown to localize mainly in cytoplasm when they were exogenously added to the cell culture medium (Yang et al. 2002). In case of AP-2, it has been displayed to localize in endosomes, a large network of cytoplasmic vesicles, after transduction (Bertrand et al. 2011). Notably, the transduction of both fusion proteins was time-independent from 30 min up to 2 h of incubation period. Prior study revealed that TAT-mediated protein transduction achieves maximum intracellular concentrations in less than 5 min (Becker-Hapak et al. 2001). This indicates that transduction of SOD2-TAT reached maximum intracellular concentration before 30 min. In addition, it has been previously reported that ANG1005, an AP-2 conjugated with three molecules of paclitaxel, was initially uptaken into U87 human glioblastoma cells within 2 min and obtained to the saturation point after 15 min (Bertrand et al. 2011). Altogether, these reasons can explain why the intracellular fluorescence intensities were not markedly increased in a time-dependent manner in our study. However, the difference in transduction period of each fusion protein may be due to the properties of transduced fusion protein, such as the degree of unfolding, polarity and the molecular shape of the protein (Bertrand et al. 2011). To perform cell viability assay, all purified enzymes were used in units of specific enzymatic activity instead of molar concentration since estimated 50 % reduced activity of fusion proteins. Our results demonstrated that pretreatment with SOD2-TAT could protect the bEnd.3 cell death caused by paraquat up to 4 mM and pretreatment with SOD2-AP-2 dramatically increased cell viability up to 2 mM. These results suggest that the intracellular level of transduced SOD2-TAT was greater than that of transduced SOD2-AP-2 affecting to the better protection of SOD2-TAT at high concentration of paraquat. It has been shown that TAT peptide can be transcytosed in cells by adsorptive-mediated transcytosis (AMT) without specific receptor binding (Herve et al. 2008). In contrast, AP-2 peptide is transported to cells via a specific and saturable receptor-mediated transcytosis (RMT) that requires the interaction with LRP1 receptor (Bertrand et al. 2011). Therefore, the amount of transduced SOD2-AP-2 is restricted by the number of LRP1 on the BMVECs, while the amount of transduced SOD2-TAT is higher due to independently cellular uptake. Although, our results indicate that SOD2-TAT conferred more protection against paraquat as compared with SOD2-AP-2, it is noteworthy that use of SOD2-AP-2 provides two competitive advantages: (1) AP-2 peptide is more specific and selective for brain endothelial cells than TAT peptide which entering to all cell types and (2) AP-2 lacks of immunogenicity but TAT has been previously reported about its toxicity and immunogenicity (Trehin and Merkle 2004).

In summary, we not only demonstrate that the exogenous human MnSOD delivery to bEnd.3 cells through the AP-2 peptide is feasible, but also show that this fusion protein confers the protection against oxidative stress. Our findings may provide a new strategy for protecting against BMVECs destruction resulted from oxidative damage and an opportunity for the treatment of various oxidative stress-related neurological diseases. Although, many studies have tested and shown the success of delivery system across the BBB using AP-2 peptide conjugated with various molecules, such as drugs or nanoparticles in animal models (Demeule et al. 2008a, 2008b; Bertrand et al. 2010, 2011; Xin et al. 2012). However, application of AP-2-mediated protein delivery has never been investigated in vivo yet. Further studies in physiological and pharmacological aspects in animal model will be necessary to establish the efficacy and potential use of SOD2-AP-2 as a therapeutic agent for neurological diseases. Therefore, the study of in vivo protective function using an animal model will be further elucidated.

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Conflict of Interest Warawan Eiamphungporn, Sakda Yainoy and Virapong Prachayasittikul declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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